

Structure and Localization of Genes Encoding Aberrant and Normal Epidermal Growth Factor Receptor RNAs from A431 Human Carcinoma Cells

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A431 cells have an amplification of the epidermal growth factor (EGF) receptor gene, the cellular homolog of the *v-erb B* oncogene, and overproduce an aberrant 2.9-kilobase RNA that encodes a portion of the EGF receptor. A cDNA (pE15) for the aberrant RNA was cloned, sequenced, and used to analyze genomic DNA blots from A431 and normal cells. These data indicate that the aberrant RNA is created by a gene rearrangement within chromosome 7, resulting in a fusion of the 5' portion of the EGF receptor gene to an unidentified region of genomic DNA. The unidentified sequences are amplified to about the same degree (20- to 30-fold) as the EGF receptor sequences. In situ hybridization to chromosomes from normal cells and A431 cells show that both the EGF receptor gene and the unidentified DNA are localized to the p14-p12 region of chromosome 7. By using cDNA fragments to probe DNA blots from mouse-A431 somatic cell hybrids, the rearranged receptor gene was shown to be associated with translocation chromosome M4.

Epidermal growth factor (EGF) is one of a number of peptides that stimulate cell growth by binding to high-affinity cell surface receptors (46). The EGF receptor is a membrane-spanning glycoprotein with a molecular weight of 170,000 (8, 14, 43). The receptor consists of two separate domains: an external amino portion which is glycosylated and binds EGF and a cytoplasmic carboxylated portion which contains EGF-dependent protein kinase activity capable of phosphorylating itself and other substrates at specific tyrosine residues (8, 28, 29). These two domains are connected by a hydrophobic transmembrane-spanning region (57, 61). EGF and its receptor are cointernalized via surface membrane-coated pits and are ultimately delivered to lysosomes where they are degraded (5, 60). The role of internalization in EGF action and the mechanism by which EGF elicits a wide variety of cellular responses and stimulates cell proliferation is still unknown (11, 26, 32).

Interest in the role that growth factors play in regulating cellular growth has increased dramatically with the discovery that the simian sarcoma virus transforming protein p28^{sis} is nearly identical to one of the two polypeptide chains of the human platelet-derived growth factors (15, 58). More recently, peptides of the human EGF receptor have been isolated, sequenced, and found to be homologous to the avian erythroblastosis virus *erb B* oncogene product (17, 64). Avian erythroblastosis virus is a retrovirus known to induce sarcomas and erythroblastosis in birds and transformation in cultured cells (18, 21, 65). The human EGF receptor gene appears to be the *c-erb B* protooncogene; both have been localized to the same general region of chromosome 7 (30, 52).

Oncogenes of malignant cells have been found to be

associated with a variety of abnormalities. A large number of tumor cells have been shown to harbor amplified oncogenes, such as *c-myc*, *c-ras*, and *c-abl* (2, 9, 10, 13, 47, 48). Many of these exhibit enhanced oncogene expression in the form of RNA and protein overproduction. In addition, transformed cells expressing high levels of either *myc* or *myb* protein were found to possess RNA species of unusual size (3, 35, 39, 40, 48, 53) and to contain rearrangements in the vicinity of the protooncogene. Of the 14 *c*-oncogenes whose chromosomal location has been identified, at least half have been found at sites involved in chromosomal abnormalities (42). The breakpoints of a number of these rearrangements have been cloned and shown to contain protooncogenes such as *c-abl* in chronic myeloid leukemia and *c-myc* in Burkitt lymphoma (1, 12, 25, 54, 56).

The A431 epidermoid carcinoma cell line contains very large numbers of EGF receptors (19), which are encoded on genes exhibiting many of the characteristics typical of oncogenes in tumor cells. We and others have demonstrated that A431 cells have an EGF receptor gene copy number that is amplified ~30-fold (33, 37, 57). In addition, A431 cells secrete a truncated form of the EGF receptor (36, 59, 62) and contain an aberrant 2.8- to 2.9-kilobase (kb) EGF receptor RNA which diverges from EGF receptor RNAs found in other cell types (10 and 5.6 kb) at its extreme 3' end (33, 37, 57, 61). A431 cells are hypotetraploid, containing two normal 7 chromosomes, and at least two 7 translocation marker chromosomes (M4 and M14) (50). These translocation chromosomes could be the source of the shortened 2.9-kb RNA. To investigate the origin of the aberrant RNA and whether its presence is in any way responsible for the initiation or maintenance of the transformed phenotype of A431 cells, a cDNA encoding this RNA was isolated, analyzed, and used

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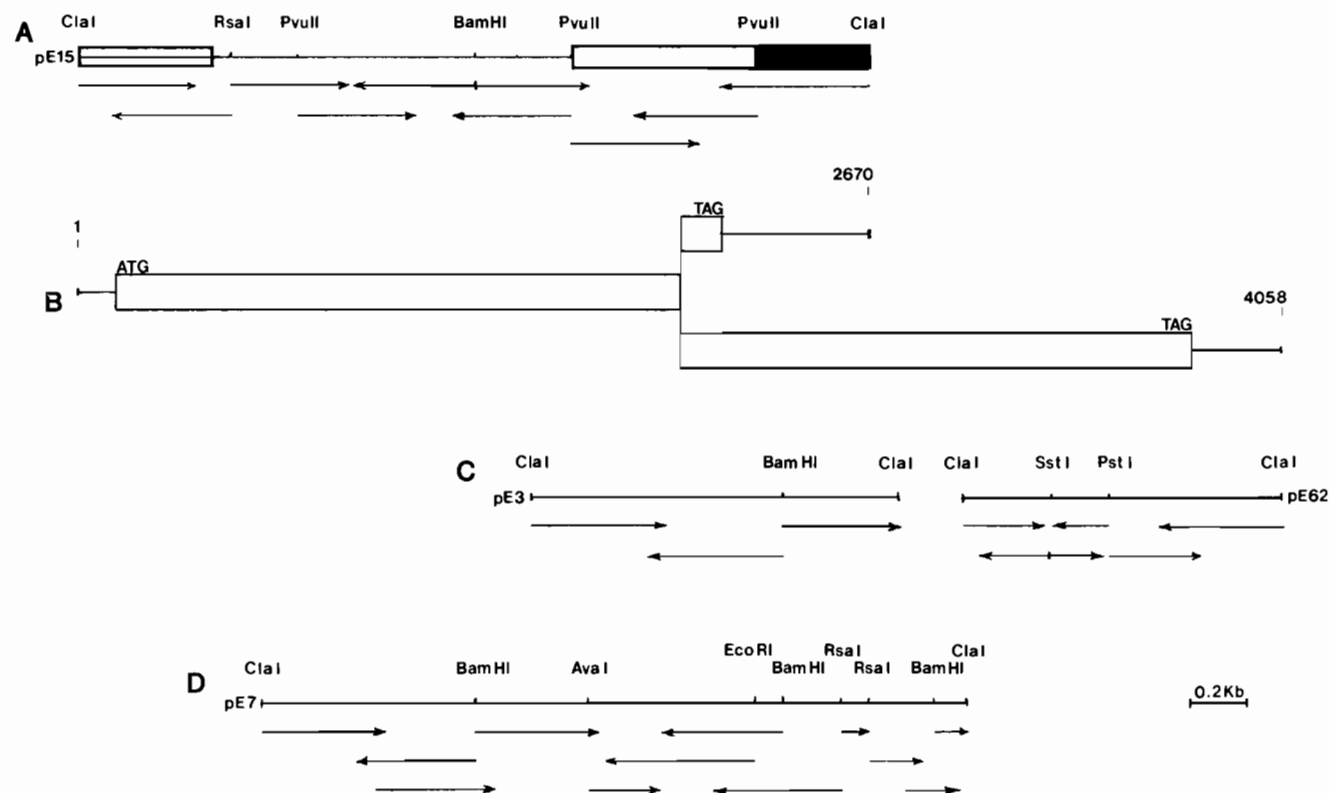


FIG. 1. Dideoxynucleotide sequencing strategy of four isolated A431 EGF receptor cDNA clones. cDNA inserts shown are pE15 (A); pE3 and pE62 (C); and pE7 (D). Restriction enzyme sites used for sequencing are indicated. Arrows indicate the direction of sequencing (label is at the tail). The map of the cDNA insert of plasmid pE15 (A) also includes boxed areas representing probes used in this study: lined box, 450-bp *ClaI*-*EcoRI* 5' fragment; open box, 600-bp *PvuII* breakpoint-spanning fragment; closed box, 400-bp *PvuII*-*ClaI* fragment specific for the rearranged DNA juxtaposed to the EGF receptor gene in A431 cells. (B) Structure of two A431 RNAs (short, aberrant, 2.9 kb; normal, 5.6 kb), including the coding regions (large open areas bordered by an ATG translation start signal and TAG stop signals), and the location of the breakpoint at which the 2.9-kb RNA diverges from the normal EGF receptor RNA (vertical line).

as a hybridization probe to examine the structure of the gene from which it was transcribed.

MATERIALS AND METHODS

Cell culture. Cell lines were maintained as described previously (62). The ovarian carcinoma cell lines were 2780 and 1847 (S. Aaronson, National Institutes of Health) and OVCA2 and OVCA3 (T. Hamilton and R. Ozols, National Institutes of Health). A431 and the kidney carcinoma cell line A498 were obtained from G. Todaro (National Institutes of Health). WI38 are normal human embryo fibroblasts.

cDNA cloning and sequencing. A cDNA library was made with polyadenylated [poly(A)⁺] RNA from A431 human epidermoid carcinoma cells used as a template for reverse transcriptase, and the resulting double-stranded cDNA was ligated into pBR322 with *ClaI* linkers (61). The library was screened with a 290-base-pair (bp) *ClaI*-*PstI* 5' fragment from the EGF receptor-encoding plasmid pE7 (61) to isolate a cDNA encoding the aberrant 2.9-kb RNA. One positive clone (plasmid pE15) was found to contain a 2.7-kilobase-pair (kbp) insert. Plasmid DNA was purified by CsCl centrifugation. The pE15 insert was sequenced by the dideoxynucleotide chain termination method described previously (44, 45, 61). cDNA fragments were ligated into appropriate molecular cloning sites of bacteriophage M13 (38), which was transfected into *Escherichia coli* JM-101 and isolated as single-stranded recombinant phage DNA.

RNA isolation and blotting. Total RNA was isolated by solubilizing A431 or KB cultured cells in guanidine isothiocyanate and by centrifuging the resulting extract over a CsCl cushion (7). Oligodeoxythymidylate-cellulose chromatography was used to isolate poly(A)⁺ RNA. RNA (5 μ g) was fractionated over 1% agarose-formaldehyde and transferred to nitrocellulose (55). Prehybridization, hybridization, and washing were carried out as described previously (55, 61). Labeled DNA probes were prepared by nick-translation by the method of Maniatis et al. (34).

Genomic DNA isolation and blotting. High-molecular-weight genomic DNA was isolated by sodium dodecyl sulfate-proteinase K cell lysis, phenol-chloroform extraction, and exhaustive dialysis (37, 62). Genomic DNA (10 or 20 μ g) was digested with the appropriate restriction enzyme, electrophoretically fractionated on 1% agarose, transferred to nitrocellulose (51), and hybridized to nick-translated cDNA fragments. To reduce probe-related nonspecific background, all ³²P-labeled probes were first exposed to prebaked blank nitrocellulose filters in complete hybridization buffer containing 10% (wt/vol) dextran sulfate for 2 h before hybridization to the experimental DNA filter. Hybridization and washing were as described previously (37), and washing was carried out for 1 h at 60°C in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.2% sodium dodecyl sulfate.

Creation of somatic cell hybrids. Somatic cell hybrids between mouse A9 cells and human A431 cells were created

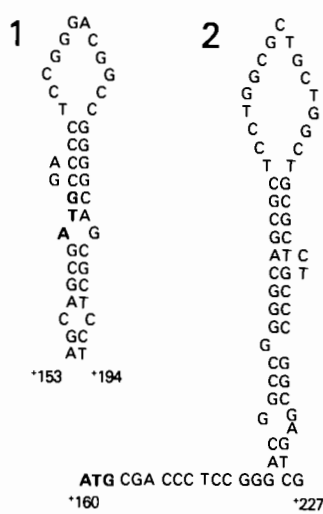


FIG. 3. Two possible hairpin structures which could form at the 5' end of EGF receptor cDNA or mRNA. The numbers at the bottom of each structure correspond to those found in the sequence shown in Fig. 2. The putative translation start site (ATG) is shown in boldface type. Note that the two loops are mutually exclusive. The free energies of formation of the two loops are -17.0 (1) and -28.4 (2).

with polyethylene glycol as described previously (50). The properties and maintenance of two of the resulting hybrids (AA-1 and AA-7) have been described by Shimizu et al. (50). The C2B5 hybrid cells were clones from the AUG series of human-mouse hybrid cells, which are formed by fusing

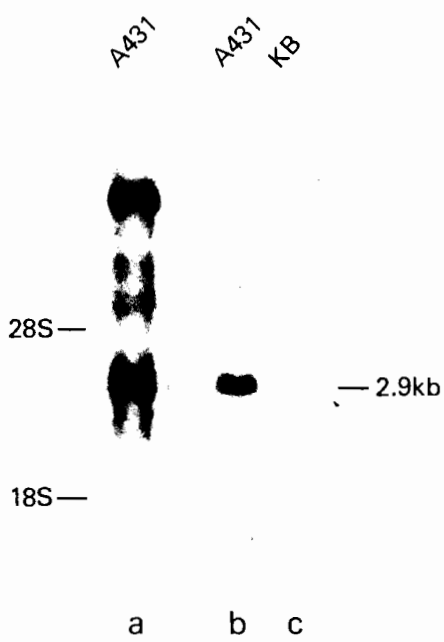


FIG. 4. Examination of pE15-specific RNAs by RNA blot analysis. Either the 450-bp *Clal-EcoRI* cDNA fragment from the 5' portion of pE15 (lane a) or a 400-bp *PvuII-Clal* cDNA fragment from the 3' portion of pE15 (lanes b and c) (Fig. 1A) was used as a probe. Poly(A)⁺ RNA was isolated from either A431 cells (lanes a and b) or KB cells (lane c), electrophoretically fractionated on agarose, transferred to nitrocellulose, hybridized to the appropriate probe, and subjected to autoradiographic analysis. The position of rRNA markers is shown on the left.

mouse A9 with human diploid fibroblasts carrying an X/7 chromosome translocation (49).

Chromosome in situ hybridization. DNA fragments to be used as hybridization probes for in situ hybridization were nick-translated (34) with ³H-labeled deoxynucleotides to a specific activity of between 5×10^6 and 1×10^7 cpm/ μ g, purified over a Sephadex G-100 column, and used directly. The in situ hybridization studies were performed according to previously published methods (22).

RESULTS

Isolation of cDNA encoding the aberrant RNA. Previously, we had isolated a 2.4-kbp cDNA (pE7) encoding the EGF receptor from an A431 cDNA library (61). The 5' portion of this cDNA is homologous to the 5' end of a 2.9-kb A431-specific EGF receptor-like RNA when analyzed by R-looping and RNA (Northern) blotting (61). Therefore, a fragment from the 5' end of pE7 (a 290-bp *Clal-PstI* fragment) was used to rescreen the A431 cDNA library, and a plasmid

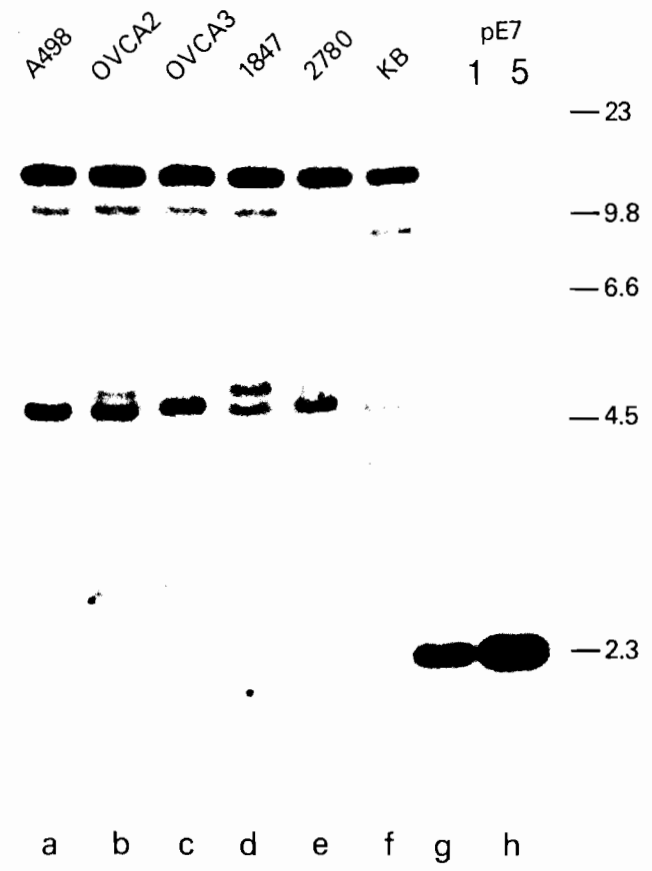


FIG. 5. Determination of EGF receptor gene copy number by DNA blot analysis. (Lanes a through f) Genomic DNA was isolated from the indicated cell lines, digested with *HindIII*, fractionated, transferred to nitrocellulose, hybridized to ³²P-labeled pE7 cDNA, and autoradiographed. (Lanes g and h) Plasmid pE7 was digested with *Clal* to release the 2.4-kbp cDNA insert, diluted, and loaded at the equivalent of one or five copies per haploid genome. Numbers on the right (in kbp) mark the position of λ -*HindIII* standards. The 2.7-kbp EGF receptor genomic DNA fragment reported to be absent from WI38 cells (37) in fact was found in several other cell lines examined (lanes a through f) and therefore is not unique to A431 cells.

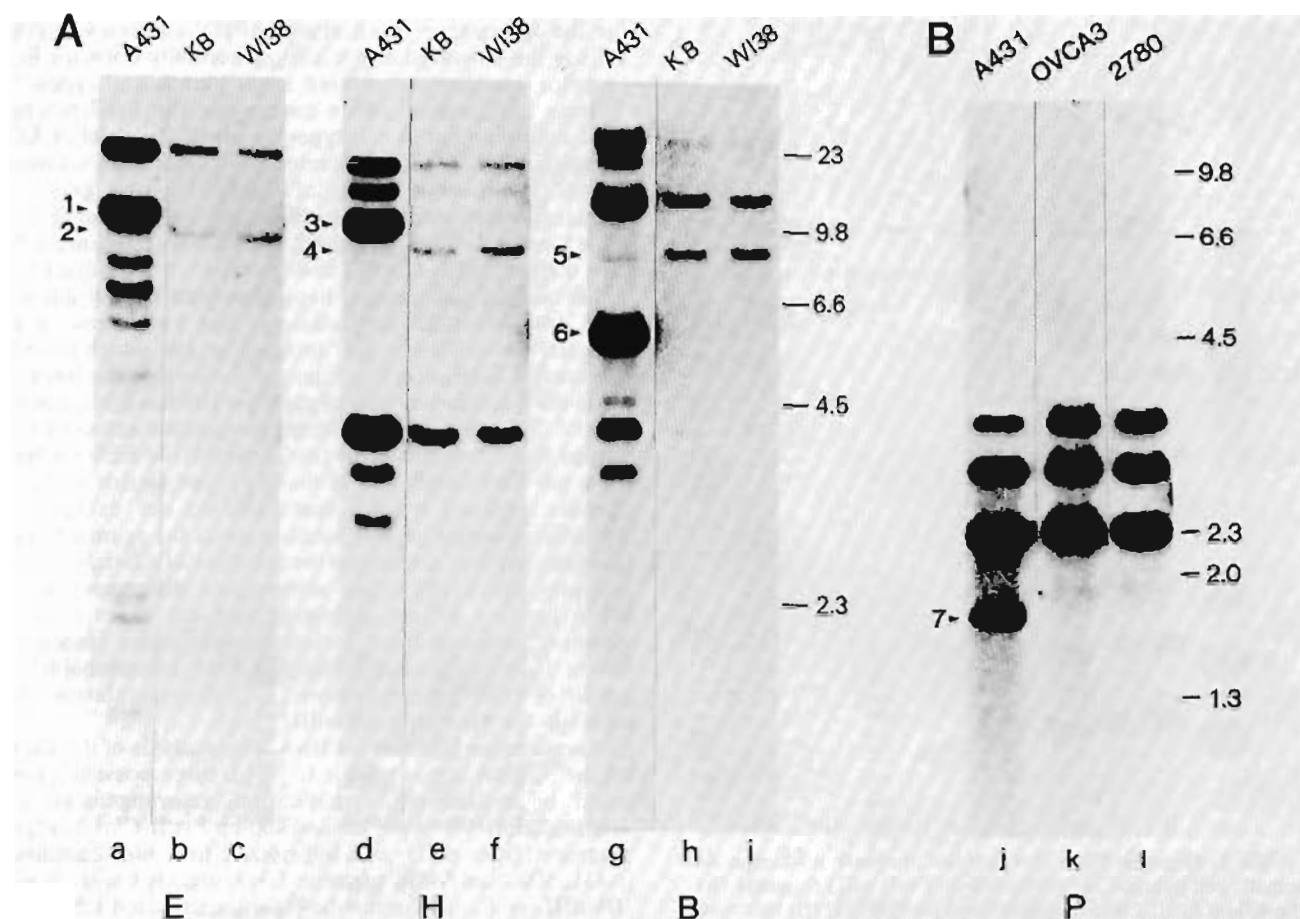


FIG. 6. Examination of the 3' portion of the gene encoding the 2.9-kb EGF receptor-related RNA by DNA blot analysis. Either the 400-bp *PvuII*-*ClaI* fragment of pE15 (A) or the 600-bp *PvuII* fragment of pE15 (B) (Fig. 1A) was used as a probe. Genomic DNA (10 μ g, lanes a through i, k, and l; 1 μ g, lane j), isolated from the cell types indicated, was digested with *EcoRI* (E; lanes a through c), *HindIII* (H; lanes d through f), *BamHI* (B; lanes g through i), or *PvuII* (P; lanes j through l) and analyzed by Southern blotting and autoradiography. Marker numbers on the right are in kbp. The arrowheads labeled 1 through 7 are described in the text. Minor A431 bands in (A) are known to be due to a slight contamination of the probe with EGF receptor-specific cDNA sequences from neighboring restriction endonuclease fragments of pE15. In (A) the lanes containing A431 DNA (a, d, and g) are presented as a slightly shorter exposure relative to other lanes (two- to threefold) to make comparison between cell types easier. On longer exposure, bands 2, 4, and 5 are visible in A431 DNA. In (B) about one-tenth the amount of A431 DNA was loaded (lane j).

containing the largest insert (pE15) was selected for further analysis. Plasmid pE15 contains 2.65 kbp of insert flanked by artificial *ClaI* sites. Figure 1A shows the overall structure of pE15, including a partial restriction map indicating the DNA fragments used as hybridization probes in this study. Figure 2 contains the cDNA sequences representing the pE15-specific aberrant RNA (S), as well as the normal EGF receptor RNA (L). The strategy for the sequencing of the various cDNAs is shown in Fig. 1. The 5' portion, which is 2.1 kbp in length, is identical to the normal EGF receptor RNA (Fig. 2, L&S) as described previously (57, 61). However, the sequence of 600 bp at the 3' end diverged completely from the normal EGF receptor sequences (Fig. 1B; Fig. 2, S). In Fig. 2 (L) are also shown the location in the normal EGF receptor of the major tyrosine autophosphorylation site (16), the nucleotide binding domain (4, 27), and the threonine phosphorylated by C kinase (29). The divergent sequence beginning at nucleotide 2079 in the pE15 insert contains none of these structures (Fig. 2, S). An almost identical clone has been isolated and sequenced by Ullrich et al. (57).

Structure of the pE15 cDNA. Examination of the sequence revealed that the transmembrane-spanning region of the EGF receptor was missing in the 2.7-kbp pE15 cDNA insert (Fig. 2). This finding suggests that the shortened RNA may encode the A431-specific secreted EGF receptor-like protein. This secreted protein would not be expected to contain the hydrophobic membrane-spanning region. In place of this region there is an open reading frame encoding 17 amino acids followed by a stop signal and a stretch of 3' noncoding sequences.

Another interesting property of EGF receptor cDNA is the potential of the sequences at the 5' end to form two mutually exclusive hairpin loop structures (Fig. 3); only one of these (loop 1) contains the putative AUG start codon within the stem. If these structures actually exist in the RNA in vivo, one possible consequence of a transition between the two loops could be a translation on and off switch. AUG start codons have been localized to similar hairpin structures in three chicken collagen RNAs (63).

The 600 bp at the 3' end of the pE15 insert, which does not code for the EGF receptor, provides a tool to investigate the

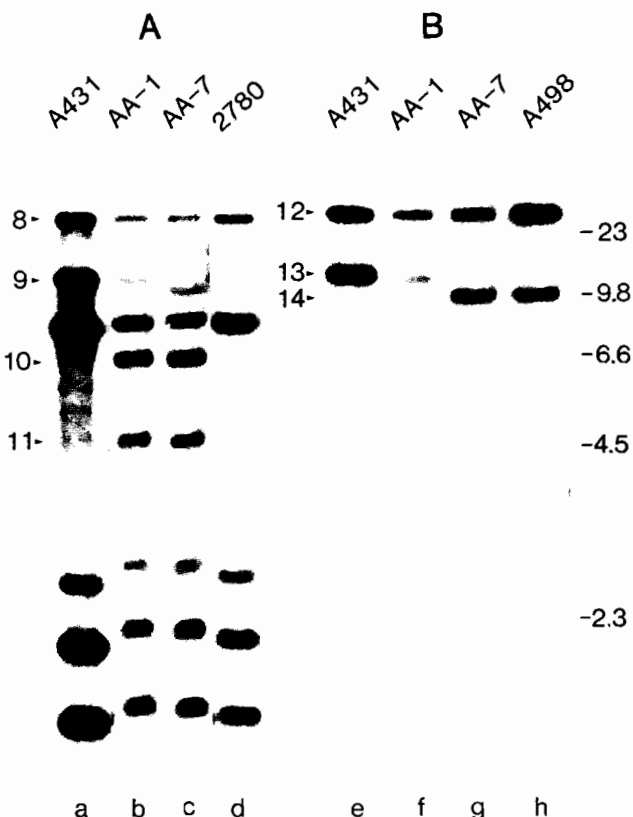


FIG. 7. Genomic DNA blot analysis of mouse A9-human A431 somatic cell hybrids. Either the 600-bp *PvuII* pE15 fragment (A) or the 400-bp *PvuII-Clal* DNA subcloned into pBR322 (B) was used as a probe. To help equalize the hybridization signals, 1 μ g (lanes a and e), 10 μ g (lanes d and h), or 20 μ g (lanes b, c, f, and g) of genomic DNA was digested with *EcoRI*, fractionated, blotted, hybridized, and autoradiographed. Numbers on the right are in kbp. Arrowheads (8, 9, 12 through 14) are discussed in the text (8 = 12; 9 = 13). Numbers 10 and 11 in (A) most likely represent mouse EGF receptor gene fragments which cross-hybridize to the human 600-bp probe. The 400-bp probe used in (B) did not hybridize to any mouse parental A9 genomic DNA (data not shown). AA-1 contains the human chromosomes M4 and X; AA-7 contains human chromosomes 7, 10, 12, and X. The ~10-kbp AA-7 band that hybridizes to the 600-bp *PvuII* probe (lane c) is probably not the 10-kbp AA-7 band that hybridizes to the 400-bp *PvuII-Clal* probe (lane g) because the former is not found in other cell lines (i.e., 2780 in lane d), whereas the latter clearly is (i.e., A498 in lane h, and 2780 [data not shown]).

origin of the 2.9-kb RNA. A 400-bp *PvuII-Clal* 3' fragment isolated from within the 600 bp of unique DNA of pE15 (Fig. 1A) was used as a probe to hybridize to poly(A)⁺ RNA from A431 cells or human KB carcinoma cells (Fig. 4). This labeled fragment hybridizes very strongly and specifically to the 2.9-kb RNA from A431 cells (Fig. 4b). KB cells contain no detectable hybridizable RNA at this autoradiographic exposure (Fig. 4c). It is not yet known whether other cell types make RNAs homologous to this 600-bp region, although this is a possibility which has been suggested previously (57). A fragment from the 5' end of the pE15 insert hybridizes to all of the major A431 EGF receptor-related RNA species (Fig. 4a). These data confirm previous results obtained from R-loop analysis of A431 poly(A)⁺ RNA (61).

EGF receptor gene copy number. One possible explanation

for the appearance of an abnormal RNA species in A431 cells is the enhanced expression of normally dormant EGF receptor pseudogenes present in all human cell types. To address this possibility, we determined the EGF receptor gene copy number in cell types in which the level of EGF receptor mRNA was not elevated. DNA blot analysis would reveal whether a family of EGF receptor genes or pseudogenes exists in the genome which might be responsible for the synthesis of the 2.9-kb RNA. Genomic DNA was digested with *HindIII* and electrophoresed adjacent to either one or five copies per haploid genome of *Clal*-digested pE7, transferred to nitrocellulose, and hybridized to the nick-translated 2.4-kbp pE7 insert (Fig. 1D) which encodes the normal EGF receptor. Figure 5 shows that the intensity of all the bands in any lane of genomic DNA is fairly close to that of one copy of pE7. Microdensitometric scanning confirmed that there are one or two copies of the EGF receptor gene per haploid genome of the cell types shown in Fig. 5. Results obtained by this technique do not exclude the possible existence of one pseudogene in the normal human genome, but they do rule out the presence of a family of EGF receptor genes. We noted some minor differences in the DNA banding pattern among several cell types (Fig. 5); however, some of these variations were found among different individuals, suggesting that they are probably the result of restriction enzyme-specific polymorphisms, particularly for the enzyme *HindIII*.

Amplification of divergent DNA. For analysis of the nature of the 3' DNA region unique to pE15, three possible events must be considered: amplification, rearrangement, and translocation. When the labeled 400-bp *PvuII-Clal* divergent fragment from pE15 was hybridized to a blot containing A431, KB, and WI38 genomic DNA digested with *EcoRI*, *HindIII*, or *BamHI*, a number of different-sized DNA fragments could be seen (Fig. 6A, lanes a through i), most of which were amplified in A431 cells to the same extent as EGF receptor gene sequences (33, 37, 57). In addition, in each of the three restriction enzyme digestions of DNA from each cell type, including A431, a minor band was present (Fig. 6A, bands 2, 4, and 5) which did not appear to be

TABLE 1. DNA blotting data of human-mouse A9 somatic cell hybrids

Cell line	Human chromosomes ^a	Genomic fragments specific for divergent region (kbp) ^b	
		<i>EcoRI</i>	<i>HindIII</i>
A431	All + M4, M14	25, 12.5, 10.5 ^c	23, 12, 9.4 ^c , 4.0
AA-1	M4, X	25, 12.5	23, 12, 4.0
AA-7	7, 10, 12, X	25, 10.5	23, 9.4, 4.0
C2B5 ^d	t(7; X)	25, 10.5	23, 9.4, 4.0
Human controls ^e	All	25, 10.5	23, 9.4, 4.0
Mouse A9	None	None	None

^a Chromosomal content of A431 and hybrid cell lines are from Shimizu et al. (49, 50). M4 and M14 are marker chromosomes containing a translocated chromosome 7.

^b Numbers represent any fragments capable of hybridizing to the subcloned 400-bp *PvuII-Clal* DNA or to the 600-bp *PvuII* breakpoint-spanning DNA. Both are known to be specific for the 3' divergent region of pE15, which encodes the aberrant 2.9-kb mRNA. In this study *EcoRI* fragment 25 is bands 8 and 12; fragment 12.5 is bands 9 and 13; fragment 10.5 is bands 2 and 14. *HindIII* fragment 12 is band 3; fragment 9.4 is band 4 (see Fig. 6 and 8).

^c These two bands are detectable in A431 cells, but they are not amplified.

^d From Hunts et al., in preparation.

^e A variety of normal and malignant cell lines were used as unrearranged human controls (see text).

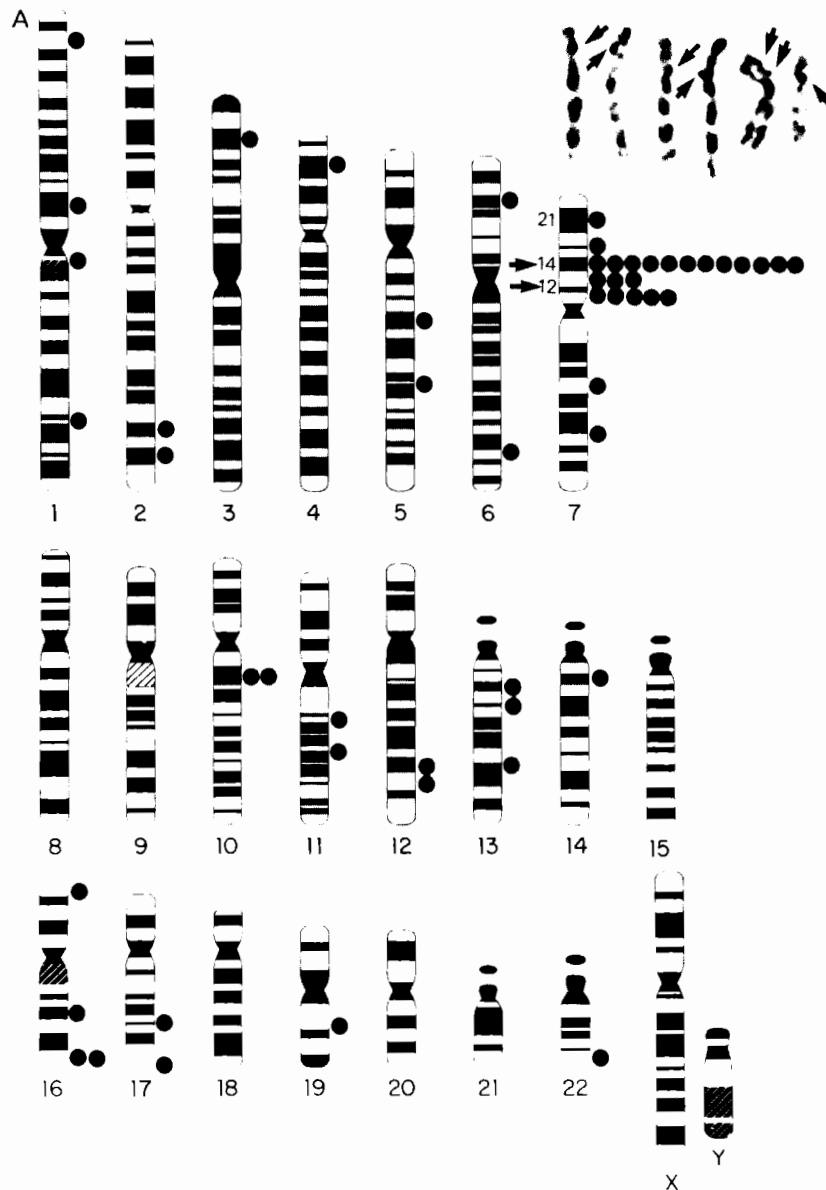


FIG. 8. (A) Schematic representation of MTX-synchronized Wright-stained metaphase chromosomes depicting all chromosomal grains observed in 37 cells in in situ hybridization with a ^3H -labeled, 2.4-kbp insert of plasmid pE7; 39% of the total grains were observed at bands 7p12-7p14. In the upper right corner of the figure is shown six individual chromosome 7s (from different metaphases) with arrows indicating the individual grains. (B) Distribution of grains observed on designated chromosomes in in situ hybridization of line A431 (representative karyotype); 34 of the 82 grains (41%) were localized to markers A and C. Light arrows indicate abnormal chromosomes. Marker chromosomes are designated A through E. The bottom row shows the marker chromosomes from nine different in situ preparations, and the dark arrows indicate the grain locations.

amplified in A431, and was not always observed with other restriction enzymes and other probes. The unamplified fragments probably represent cross-hybridization with DNA from another region of the genome.

Evidence for EGF receptor gene rearrangement. Figure 6A also shows that when genomic DNA is restricted with *EcoRI*, *HindIII*, or *BamHI* and hybridized with the 400-bp *PvuII*-*Clal* fragment, highly intense, amplified bands of 12.5, 12.0, and 6.1 kbp, respectively, appear in A431 DNA (Fig. 6A, bands 1, 3, and 6) but not in either KB or WI38 DNA. These results suggest that a gene rearrangement has occurred in A431 carcinoma cells, wherein the 5' portion of the EGF receptor gene has been fused with unidentified DNA,

resulting in the appearance of unique restriction fragments characteristic of A431 DNA.

When a *PvuII* 600-bp pE15 fragment spanning the apparent breakpoint between the EGF receptor and the unidentified genomic DNA (Fig. 1A) was labeled with ^{32}P and hybridized to this filter, the same A431-specific amplified bands were seen (data not shown). When this probe was hybridized to blotted genomic DNA digested with *PvuII*, an amplified fragment unique to A431 cells was again found (Fig. 6B, band 7).

In contrast to the obvious changes observed when the 3' end of pE15 was used as a probe, little difference in the banding pattern was observed between A431 and other cell



FIG. 8—Continued

types when the EGF receptor, 450-bp *ClaI-EcoRI* 5' fragment of the pE15 insert (Fig. 1A) was hybridized to similar genomic blots. As expected, the A431 gene copy number is about 20-fold higher; otherwise, the 5' end of the EGF receptor gene in A431 cells does not seem to be substantially modified (data not shown). However, final judgment on the structure of the 5' region awaits promoter localization and analysis.

One interpretation of these data is that the A431-specific, amplified fragments (Fig. 6, bands 1, 3, 6, and 7) are generated by a chromosomal rearrangement in which the 5' portion of the EGF receptor gene has been fused with genomic DNA containing the divergent 600-bp region of pE15. These fused sequences are probably located on an abnormal chromosome (M4, M14 [50], or some other marker chromosome). Detailed information about the putative rearrangement will come from sequence analysis of a cloned breakpoint fragment of A431 genomic DNA.

Relationship between rearrangement and translocation. To determine whether the apparent rearrangement of the EGF receptor gene is found in morphologically normal chromosomes or is limited to the translocation marker chromosome

M4 of A431, interspecies somatic cell hybrids between mouse A9 and A431 cells (50) were utilized. When genomic blots containing DNA from these fused cells were hybridized to the 600-bp *PvuII* probe which spans the putative breakpoint in the 3' end of pE15, the results revealed that a band comigrating with the 12.5-kbp *EcoRI* A431-specific DNA fragment (Fig. 6A, band 1; Fig. 7A, lane a, band 9) was found in the hybrid cell line AA-1, which contains the M4 translocation chromosome (Fig. 7A, lane b, band 9). It was not found in the cell line AA-7 (Fig. 7A, lane c), which contains a small number of human chromosomes, including morphologically normal chromosome 7 (50). Similar results were obtained with the 400-bp *PvuII-ClaI* probe (Fig. 7B, band 13) and with *HindIII*-digested genomic DNA blots (autoradiographic data not shown). In Table 1 these data are summarized and provide strong evidence that the rearrangement event is associated with the translocation chromosome M4.

These mouse-A431 somatic cell hybrid experiments also help in the identification of the chromosomal origin of the unique 600-bp sequence of pE15. Figure 7A (band 8) shows that the 600-bp *PvuII* probe spanning the breakpoint hybrid-

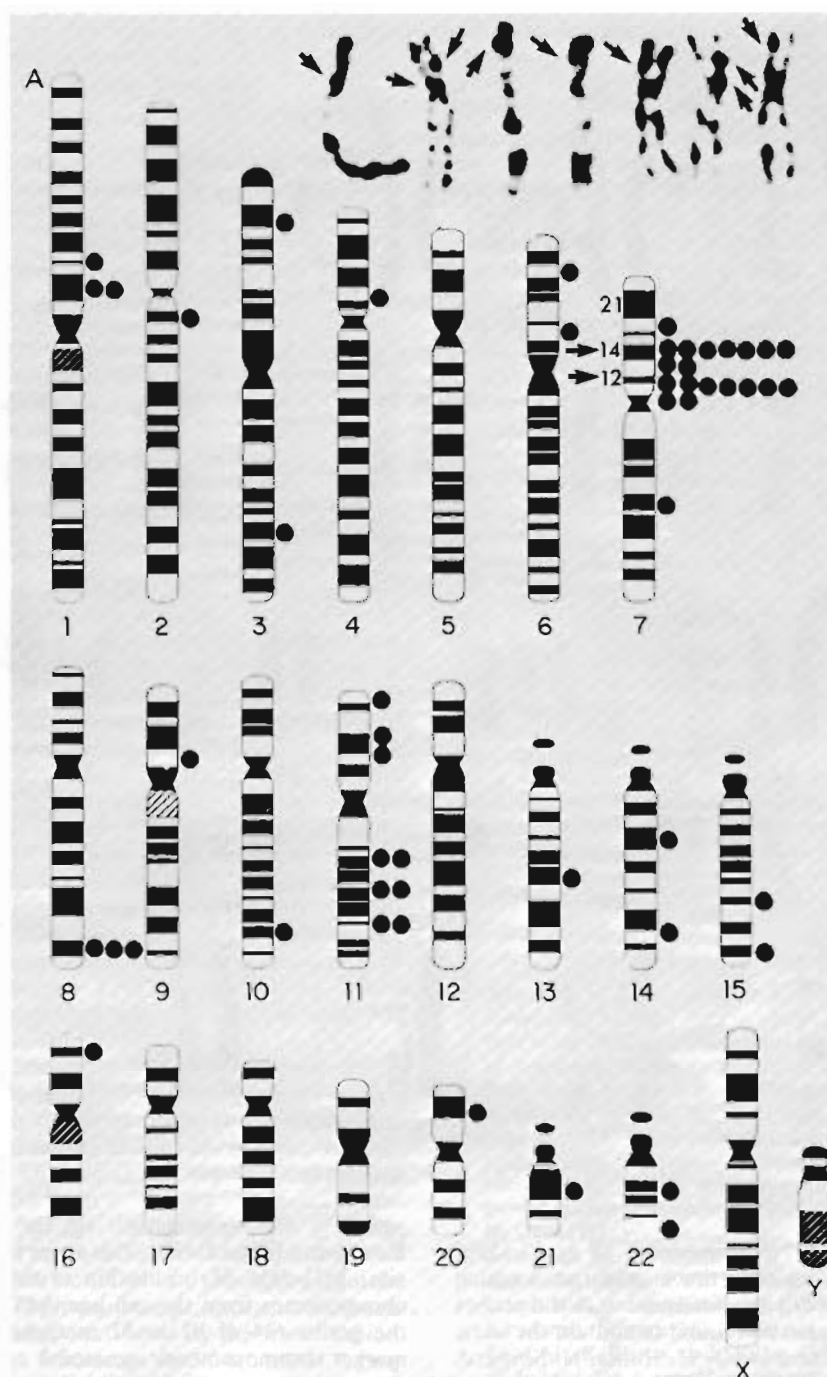


FIG. 9. (A) Schematic representation of MTX-synchronized Wright-stained metaphase chromosomes depicting all chromosomal grains observed in 35 cells with ^3H -labeled, 400-bp *PvuII*-*Clal* fragment (Fig. 1A): 19 of the 53 grains (36%) were observed at bands 7p12-7p14. In the upper right corner of the figure is shown seven chromosome 7s from six individual cells with arrows indicating the individual grains. (B) Distribution of grains observed on designated chromosomes in A431: 24 of the 66 grains (36%) were localized on markers A and C. Arrows indicate abnormal chromosomes.

ized to the same 25-kbp *EcoRI* fragment from both AA-1- and AA-7-digested genomic DNA (lanes b, c), as well as from an unrearranged control cell line (lane d).

Figure 7B shows that when a fragment containing no EGF receptor sequences (the 400-bp *PvuII*-*Clal* DNA subcloned into pBR322) was used as a probe, two fragments, including the same 25-kbp *EcoRI* fragment as shown in Fig. 6A and 7A, were found in both a human cell line with a full

complement of chromosomes (lane h) and AA-7 containing human chromosome 7 (lane g, bands 12 and 14). These data suggest that the 600-bp divergent region of pE15 originates from chromosome 7. This conclusion was supported by blots containing genomic DNA digested by *HindIII* instead and hybridized with the probes described above (data not shown). To eliminate the possibility that genomic fragments specific for the 600-bp region of pE15 are from a chromo-



FIG. 9—Continued

some other than 7 in AA-7 (chromosome 10 or 12 [50]), another cell line possessing only the human translocation chromosome X/7 (C2B5 [49]) was analyzed with the probes from the 600-bp pE15 region and found to contain the same fragments (i.e., bands 12 and 14) (J. H. Hunts, N. Shimizu, G. T. Merlino, Y.-h. Xu, and I. Pastan, manuscript in preparation). These data (Table 1) strongly support the notion that the rearrangement occurred within chromosome 7.

In situ hybridization chromosomal localization. To confirm the chromosomal localization of the DNA sequences in the 600-bp divergent region of pE15, in situ hybridization was utilized. A ^3H -labeled, 2.4-kbp insert of plasmid pE7 encoding the normal EGF receptor mRNA was hybridized to chromosomes prepared from normal human lymphocytes to determine the location of the EGF receptor gene in normal chromosomes. Figure 8A shows the distribution of grains observed over all the designated chromosomes: 22 of 54 (41%) grains from 37 metaphases were localized to 7p, with 91% of these grains being observed in the p12-p14 region. These results are in agreement with those described by

Kondo and Shimizu (30). This is very near a heritable fragile site, 7p11 (31). Hybridization of this probe to metaphase chromosomes from the cell line A431 revealed that 41% of the grains (34 of 82 in 32 metaphases) localized to two marker chromosomes designated A and C (Fig. 8B). The M4 chromosome described by Shimizu et al. (50) was not found in our A431 cells; apparently, there is significant instability in the chromosome population, and other marker chromosomes contain the amplified, rearranged EGF receptor genes.

When the ^3H -labeled, 400-bp *PvuII*-*Clal* fragment was hybridized to similar normal chromosome spreads, a high percentage of the grains (36% in 35 metaphases) was again localized to the p12-p14 region of chromosome 7 (Fig. 9A). When this probe was hybridized to chromosomes from A431 metaphases, 36% of the grains (24 of 66 in 30 metaphases) was again found to be localized to markers A and C (Fig. 9B). These data confirm that the alterations specifically associated with A431 genomic DNA result from a rearrangement between the EGF receptor gene and another piece of

genomic DNA residing on chromosome 7 and suggest that translocation chromosome markers such as A and C harbor many copies of EGF receptor gene sequences.

DISCUSSION

We isolated a cloned cDNA plasmid encoding the 2.9-kb aberrant EGF receptor RNA found in A431 cells. This RNA is a hybrid which does not contain the transmembrane-spanning region or the kinase domain, but instead possesses a divergent 600-bp sequence at its extreme 3' end and presumably encodes the EGF receptor-like protein secreted by A431 cells. The functions of the 2.9-kb mRNA and the secreted protein in A431 cells currently are unknown, although it is possible that overproduction of either the RNA or protein or both may be associated with the appearance of the transformed phenotype. By using a cDNA fragment containing these divergent sequences as a probe, evidence was found which suggests that a rearrangement of chromosome 7 led to the fusion of the EGF receptor gene and an unidentified DNA fragment, followed by amplification of the hybrid gene.

A431 cells have been shown to contain many abnormal chromosomes, including chromosome 7 translocation markers (50). The use of mouse A9-human A431 somatic cell hybrids containing either the translocation marker chromosome M4 (AA-1) or normal human chromosome 7 (AA-7) provided evidence that the rearrangement is associated only with the translocation chromosome (Fig. 7). Cells containing the M4 chromosome have been shown to produce the aberrant 2.9-kb RNA (Hunts et al., in preparation), further verifying the chromosomal origin of the A431-specific RNA. However, AA-7 contains genomic DNA capable of hybridizing to the divergent 600-bp region of pE15, suggesting that the observed rearrangement is due to an internal rearrangement in chromosome 7. This notion was supported by in situ hybridization of a probe from the divergent 600-bp DNA to normal human chromosome 7. The translocation event therefore may not be directly responsible for the observed rearrangement.

Other tumor cells are characterized by gross chromosomal abnormalities at specific locations. Rearrangements in malignant cells have been found to occur in the immediate vicinity of several c-oncogenes (1, 12, 25, 54, 56). Various abnormalities in the chromosomal regions containing protooncogenes also have been associated with overexpression of, or alterations in, the oncogene protein product. Amplification of several protooncogenes has been found to result in elevation in the level of the oncogene protein product (2, 13, 47, 48). The human *c-myc* oncogene has been found to be transcriptionally activated by the translocation of immunoglobulin gene enhancers to an upstream region (23). Both *c-myc* and *c-erb B* in chickens have been demonstrated to be activated by insertion of an avian leukosis virus long terminal repeat to an upstream region (20, 24, 41).

We have presented evidence here which suggests that the A431 EGF receptor gene is involved in a rearrangement with an unidentified piece of DNA. The nature of the DNA fused to the EGF receptor gene currently is unknown. It could be part of a gene which codes for a functional, although poorly expressed, mRNA in nonrearranged cells, because the cDNA encoding the 2.9-kb aberrant RNA contains an AATAAA polyadenylation site and a polyadenylic acid tail (57). The relocation of this DNA to a region within the EGF receptor gene may affect the expression of the resulting aberrant receptor gene at the level of transcription or translation.

It was a surprise to find that the pE7 cDNA probe hybridized in situ to both 7p14 and 7p12. This could represent two distinct hybridization sites or a technical artifact. The DNA blotting data (Fig. 5) indicate that the EGF receptor gene is present at a low copy number, but we could not distinguish between one and two copies per haploid genome. These results raise the possibility that two related genes reside at almost the same region of the short arm of chromosome 7, one of which could be a separate *c-erb B* gene or another receptor gene.

The site at which the EGF receptor is located on the short arm of chromosome 7 (p14-p12) probably constitutes an active region. A heritable fragile site is located at 7p11 (31). The short arm of chromosome 7 also has been shown to contain the human T cell receptor genes (6), which are thought to create diversity via rearrangement events. Here we report the occurrence of an apparent rearrangement at the p14-p12 region of chromosome 7. It is possible that the position of the EGF receptor gene on chromosome 7 renders it more susceptible to DNA modifications which alter the growth potential of the cell.

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